OECD DRAFT GUIDANCE DOCUMENT

Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure

INTRODUCTION

1. This document describes a honey bee brood laboratory toxicity test using repeated doses. It is based on the OECD Test Guideline 237: Honey bees (*Apis mellifera*) larval toxicity test, single exposure (1) ring tested in seven European laboratories, which is itself based on a method developed in France (2) (3) (4), and on methods described in the COLOSS Beebook (5) that provides useful guidance for honeybee testing, breeding honey bees, studying honey bee biology and understanding honey bee pests and pathogens.

2. The Guidance Document addresses the requirements formulated by the United States, Canada, and Europe (6) (7) (8) to test the toxicity of chemicals on immature honey bees by feeding larvae with spiked food under laboratory conditions. The present protocol involving repeated exposure of honeybees to chemicals will be evaluated by an international ring test in order to confirm the acceptance criteria (see paragraph 7).

3. The method aims at the determination of a No Observed Effect Concentration/Dose (NOEC/D) and if data allows EC_{50}/ED_{50} at the period of adult emergence following a repeated exposure of larvae to a test chemical (particularly active ingredient or formulation (formulated product in case of low solubility of the active ingredient). The data should be used in an appropriate honeybee brood risk assessment scheme. The test methods on honey bee larval toxicity – single and repeated exposure – complement the OECD TG 213 (9) and TG 214 (10) on young adult honey bees and should be seen as a lower tier screening test in the context of an overall risk assessment scheme for bees (6).

PRINCIPLE OF THE TEST

4. On day 1 (D1) of the study, first instar (L1) synchronised larvae (*i.e.* larvae of the same age) are taken from the comb of three colonies and individually placed into 48 well-plates where they are fed a standardized amount of artificial diet. From day 3 (D3) until day 6 (D6) of the test, the test chemical is administered daily to the larvae at a constant concentration (for each treatment level) in the diet in a range of five increasing test concentrations, or at one concentration in case of a limit test. Mortalities and other abnormal effects are recorded daily from D4 to D8 of the test, D15 and D22. The NOEC/D and the EC_{50}/ ED_{50} if data allows are determined on D22 for adult emergences.

INFORMATION ON THE TEST CHEMICAL

5. The water solubility, solubility in a solvent, and vapour pressure of the test chemical should be known. Useful information on the test chemical including its structural formula, purity, stability in water and light, and octanol-water partition coefficient (K_{ow}) should be reported. The physical appearance and

source (batch, lot number) of the test chemical should be described. Guidance for testing substances with physical-chemical properties that make them difficult to test is provided in the OECD Guidance Document, series on Testing and Assessment, No. 23 (11).

REFERENCE CHEMICAL

6. The standard reference chemical (positive control) is selected based on the mode of action of the test chemical. It is technical grade dimethoate (CAS 60-51-5) or technical grade fenoxycarb (CAS 72490-01-8) if the test chemical is an insect growth regulator (IGR) with expected effects on the development of immature bees. A preliminary experiment/ range-finding test may help to decide what is the most appropriate reference chemical (see paragraph 24). The reference chemical is tested to ensure that the test system and conditions are responsive and reliable at the constant concentration of 40 mg active ingredient (a.i.)/Kg of diet (i.e. 0.045 µg a.i./µL of diet) for dimethoate, or 0.25 mg a.i./Kg of diet (i.e. 0.28 ng a.i./µL of diet) for fenoxycarb. The table below indicates the amount of dimethoate and fenoxycarb which has to be added to the diet every day for one larva during the exposure period from D3 to D6, in order to maintain a constant concentration of the reference chemical.

Day	D3	D4	D5	D6	Total amount/larva
Amount of dimethoate (µg) added to the diet	0.9	1.3	1.8	2.2	6.2
Amount of fenoxycarb (ng) added to the diet	5.6	8.4	11.2	14	39.2

Dimethoate can be directly dissolved into water. It is recommended dissolving fenoxycarb into 0.5% acetone (e.g. 0.1 µ*L* acetone + 5.7 ng fenoxycarb for 20 µ*L** diet B, 0.6 µ*L* acetone + 34.3 ng fenoxycarb for 120 µ*L**diet C). Note: * volume of diet without solvent and chemical

TEST ACCEPTANCE CRITERIA

7. For judging the acceptance and quality of data obtained with the repeated exposure test, the following performance criteria apply:

- In the control plate(s), cumulative larval mortality from D4 to D8 should be $\leqq$15% across replicates.
- In the control plate(s), the adult emergence rate should be $\geqq$70% on D22; this value will be checked within the planned ring test (see paragraph 2).
- In the dimethoate positive control, larval mortality should be $\geqq$50% on D8. In the fenoxycarb positive control, the emergence rate should be $\leq$ 20% on D22. The planned ring test will check/investigate both values.

DESCRIPTION OF THE TEST

Apparatus

8. Larvae are reared in crystal polystyrene grafting cells (*e.g.*, ref CNE/3, NICOTPLAST Society) having an internal diameter of 9 mm and a depth of 8 mm. The cells are initially sterilised, e.g. by

immerging for 30 min in (e.g. 70%) ethanol or other sterilising solution, and then dried in a laminar-flow hood. Each cell is placed into a well of a 48-well plate. The top of the grafting cell may be maintained at the level of the plate, e.g. by placing a piece of dental roll wetted with 500 µL of the sterilising solution added with 15% weight/volume glycerol at the bottom of the wells (Figure 1).

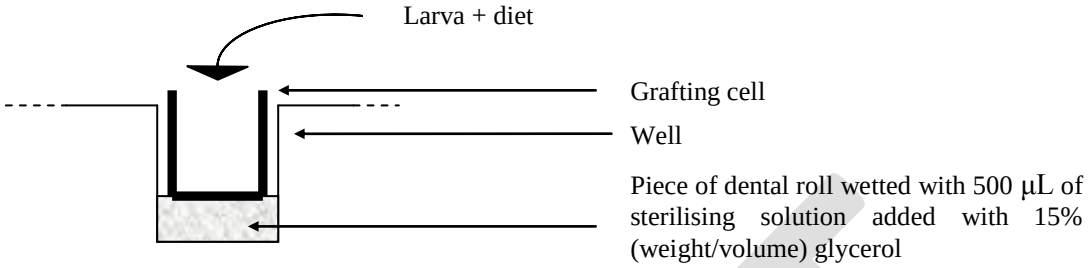

Figure 1: Larval cell in a tissue culture well

9. These plates are placed into a hermetic Plexiglas desiccator (*e.g.* NALGENE 5314-0120 or 5317-0180 according to the volume required) and kept at a relative humidity of 95% ± 5% adequate for larvae from D1 to D8 (e.g. humidity can be achieved with a dish filled with potassium sulphate (K_2SO_4) saturated solution in order to keep a water-saturated atmosphere). The desiccator is placed into an incubator equipped with a forced air circulation system at 34-35 ºC (i.e. 34.5ºC ± 0.5ºC) to equilibrate temperature around the desiccator, and as close as possible within that range for the duration of the test.

10. On D8 (pre-pupa stage), the pieces of dental roll are removed (or may be removed as it is not essential) from the wells, and after replacing the cells into the wells, the well-plates are transferred into a hermetic container at a relative humidity of 80% ± 5% adequate for pupae (e.g. humidity can be achieved with a dish filled with a saturated NaCl solution). The container is then placed into a ventilated incubator at 34-35 ºC.

11. On D15, each plate is transferred into an emergence box, e.g. a crystal polypropylene box (e.g. 11 x 15 x 12 cm) with a cover aerated e.g. with wire gauze. Emerging bees are fed with syrup/sucrose solution dispensed *ad libitum*, using bird feeders, syringes or any other suitable tool (see Figure 2). The boxes are transferred in an incubator at 34-35°C with approximately 50% of relative humidity.

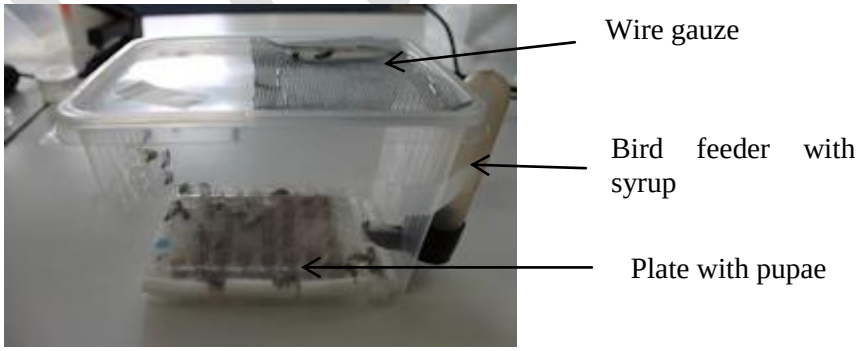

Figure 2: Example of an emergence box

Test organisms

Origin of the larvae

12. Larvae are collected from three different colonies, each one representing a replicate (see paragraph 21). Colonies should be adequately fed, all in the same healthy conditions (*i.e.* as far as possible disease- and parasite-free), with known history and physiological status.

13. Tests are conducted during the egg laying period of the queen. In case of sanitary treatment (*e.g.* mite or disease treatment measures), the date of application to the colony and the product identity are reported. No treatment with chemicals – such as antibiotics, anti-varroa, etc. – is allowed within the four weeks preceding the start of the test; healthy honeybees in the same healthy conditions should be used.

14. On D-3 (pre-phase of the test, see Figure 5), in order to ensure the production of larvae from three colonies, the queens of a minimum of three colonies are confined in their own colony in an exclusion cage containing an empty comb or a comb with emerging worker brood and empty cells (Figure 3). The exclusion cage is placed close to the combs containing brood. On D-2 (pre-phase of the test, see Figure 5), maximum 30 hours after encaging, the queen is released from the cage, after checking the presence of freshly laid eggs. Depending on the fertility of the queen, it is recommended reducing the isolation time in order to minimise the variability in egg ages. The comb containing the eggs is left in the cage, near the brood, during the incubation phase and until hatching (D1).

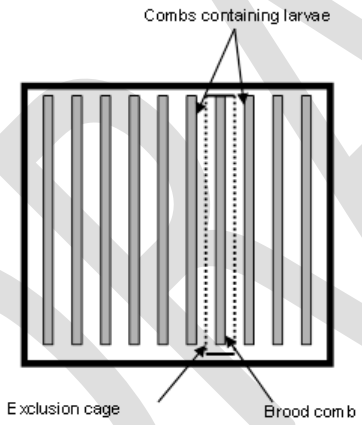

Figure 3: Cross section of a hive with an excluder cage

Preparation of rearing material

Larval food

15. The food is composed of the three following diets, adapted to the needs of the larvae at different stages of development:

- Diet A (D1): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 2% weight of yeast extract, 12% weight of glucose and 12% weight of fructose.

- Diet B (D3): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 3% weight of yeast extract, 15% weight of glucose and 15% weight of fructose.

- Diet C (from D4 to D6): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 4% weight of yeast extract, 18% weight of glucose and 18% weight of fructose.

Note: The diets A, B and C prepared in this way have a density of about 1.1 mg/µL (e.g. 20 µL diet correspond to 22 mg diet).

16. If some aggregates remain in sugar solutions they have to be completely dissolved before mixing with the royal jelly. A "fresh royal jelly" is a royal jelly collected during the preceding 12 months; it may be divided into aliquots (e.g. of approximately 5 g) in order to avoid defrosting the whole batch at each test, and stored in a freezer at ≦-10°C. Commercial sources of royal jelly might be acceptable if it can be shown that their performance compare to historical data within the testing facility, e.g. mortality does not exceed 15% during the larval period. It is recommended conducting a multi-residues analysis of each royal jelly batch in order to verify the absence of contaminants (mainly antibiotics and insecticides).

17. The diets, prepared freshly prior to each test, are stored in a fridge at ≤ +5°C (but not frozen) during the whole duration of the test. The diets may be prepared in advance and stored deep-frozen (between -18ºC and -25 ºC) until use (and if needed subsequently stored in a fridge at ≤ +5°C during the performance of the test).

Test solutions

18. The test chemical is normally dissolved or dispersed in osmosed water. For poorly soluble chemicals a solvent (e.g. acetone) may be used to prepare the stock solution. In such a case, a solvent-control – diet with the same volume of solvent as used for the treatments – is tested in addition to the regular diet-control. The volume of the organic solvent, if used, should be kept as low as possible, and in any case it should not exceed 2% of the diet final volume during the exposure period D3 to D6.

19. Dilutions of the stock solutions into the series of five test solutions are made preferably with osmosed water – or solvent for poorly soluble substances, preferably just before administration to the larvae, using disposable pipette tips equipped with a filter. The volume of the test solution mixed into the diet should not exceed 10% of the final volume if water is used to dissolve the test chemical (e.g. 2 µL of test solution for a diet volume of 20 µL on D3) or 2% if a solvent is required (e.g. 0.4 µL of test solution for a diet volume of 20 µL on D3). The test solution should be mixed into the diet in a manner that will result in even distribution of the test chemical throughout the diet.

20. A sample of the stock solution will be stored in a freezer at ≤-10°C in order to be further checked for analytical determination of the actual concentration of the test chemical. If required, the test chemical concentration may be measured in the diet (Note: the analytical measurement of the test chemical concentration in the diet is difficult because of the presence of royal jelly).

PROCEDURE

Conditions of exposure

21. The experimental unit is the individual cell containing a larva. A minimum of twelve larvae from each of three colonies are allocated, on the same plate, to each treatment level and to the control(s) and toxic reference chemical. For each test, the following treatments and control(s) are used:

- control without solvent (minimum 12 larvae X 3 colonies = 36 larvae minimum);
- control with solvent if necessary (minimum 12 larvae X 3 colonies = 36 larvae minimum);

- five treatments, *i.e.* five increasing test concentrations (each containing a minimum 12 larvae X 3 colonies = 36 larvae minimum per treatment) in a geometric series, spaced by a factor not exceeding three, and covering the NOEC/D or the EC_{50}/ED_{50}; alternatively, when a limit test is performed (see paragraph 25), a dose of 100 µg a.i. (or test chemical)/bee (equivalent to 0.064 mg a.i. (or test chemical)/kg of diet) or the maximum achievable solubility, whichever is lower, may be tested;
- a reference chemical, dimethoate 40 mg/Kg of diet or fenoxycarb 0.25 mg/Kg of diet (minimum 12 larvae X 3 colonies = 36 larvae minimum).

22. A total of seven to eight (if solvent is used) well-plates are used per test. Each group of a minimum of 12 larvae from each of the three colonies is considered a replicate for a given treatment level and identified as such on the microplate.

23. The plates are kept under dark conditions for the duration of the test. During the test, the temperature in the incubator is kept between 34ºC and 35ºC. Temporary deviations are allowed, however temperature should not drop below 23°C or go above 40°C, and these deviations should not last, as far as possible, more than30 minutes once every 24 hour.

Range-finding test

24. In order to determine the NOEC/D or the EC_{50}/ED_{50} range, it is recommended that a preliminary experiment be run with doses of the test chemical varying according to a geometrical ratio from 5 to 10. This preliminary experiment will also provide information about the mode of action of the test chemical that will help select the reference chemical (see paragraph 6).

Limit test

25. In some cases (e.g. when a test chemical is expected to be of low toxicity or is poorly soluble) a limit test may be performed using 100 µg a.i. (or test chemical)/bee (equivalent to 0.064 mg a.i. (or test chemical)/Kg of diet) or the maximum achievable solubility for poorly soluble chemicals, whichever is lower, in order to demonstrate that no effect is observed at this concentration level. Three replicates of a minimum of twelve larvae from three different colonies are used for the limit dose tested, as well as the relevant control(s), and the use of the reference chemical. If statistically significant effects occur compared to the control mortality, a full study should be conducted.

Collection of larvae

26. On D1, the comb containing first instar larvae (Figure 5) is carried from the hive to the laboratory in an insulated container in order to avoid temperature variation, and maintained at ambient temperature (not below 20ºC). It is then introduced into a laminar-flow hood or under other clean conditions for grafting. In order to avoid bias due to possible heterogeneity of the larvae, it is highly recommended selecting newly hatched larvae that have not yet formed a "C" shape, and allocating larvae randomly to the plates for each colony. A minimum of twelve larvae from each of the three replicate colonies is needed on D3 – the day of the first administration of the test chemical treatment; therefore the test may be initiated on D1 with larvae in excess of that number from each colony.

27. Alternatively, this randomised allocation of larvae may be done on D3, just before the administration of the first chemical treatment.

Grafting and feeding of larvae

28. The diet is warmed in the incubator before use. The grafting is performed preferably on a warming plate maintained at 34-35°C. The micropipettes used to place the diet into the cells are equipped with disposable tips. On day 1 (D1), a volume of 20 µL of diet A is dropped into each cell, and one larva is delicately collected from the comb and transferred to each cell, on the surface of the diet, using a grafting tool or a wetted paintbrush (e.g. No. 3/0). When a plate is completed with a minimum of 12 larvae from each colony, it is placed, as far as possible in a single layer, into the hermetic container, which has previously been placed into a ventilated incubator at 34-35°C (Figure 4), and as close as possible within that range for the duration of the test.

Figure 4: larvae incubation device

29. All larvae are fed once a day (except on D2), preferably on a warming plate that should not be warmed above 35 °C, with a sterilised transparent pipette tip with filter (to avoid contamination) or a multi stepper pipette following the schedule of Figure 3, in particular the volume of diet provided to individual larva is adapted on a daily basis. Care should be taken to avoid touching and drowning the larvae when feeding them. Food is placed close to the larva, along the wall of the grafting cell. Additional food should be added to the cell even if the previous allocation has not been totally consumed. The presence of uneaten food on D8 should be qualitatively reported.

Repeated administration of the chemical in the test solution

30. On D3, a minimum of twelve well-fed larvae from each of the three colonies are selected. From D3 to D6, larvae are fed with diets (diet B at D3, and diet C from D4 to D6) containing the test solution at the suitable concentration. The mixing of the test solution with the diet is performed just before administration, unless the stability of the test chemical in the diet has been demonstrated and is reported. From D3 to D6, for each treatment, a different micropipette tip with filter is used to administer the diet containing the treatment in order to avoid contamination.

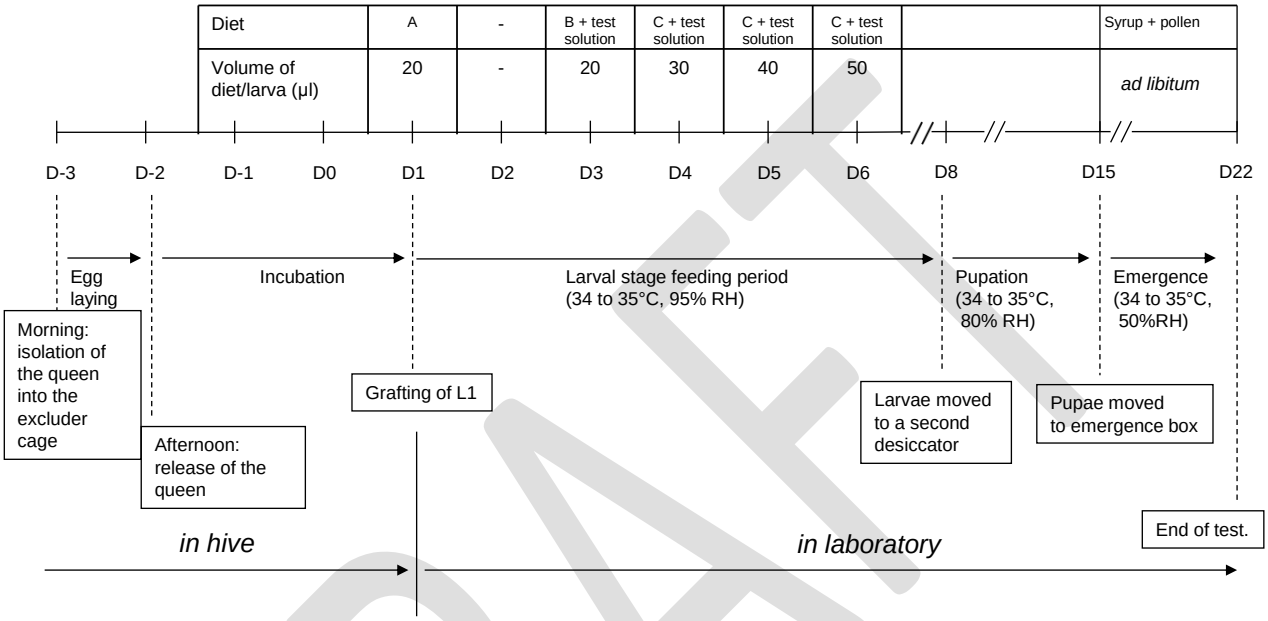

Figure 5: Schematic representation of the important steps of the larval repeated exposure toxicity test (D = day; RH = relative humidity)

Termination of the test

31. On D22, the number of emerged adults and non-emerged bees are counted (see paragraph 33) and the test is terminated by freezing the plates at $\leq$ -10°C.

Observations

32. Following the first chemical exposure on D3, mortalities are checked and recorded at the time of feeding from D4 to D8 and D15. An immobile larva or a larva which does not react to the contact of the grafting tool or paintbrush is noted as dead. On D15, larvae that have not transformed into pupae are recorded as dead (see Annex) and removed. Hatched adults – i.e. alive adult bees and dead adults which have left their cell and show a normal development – are recorded on D22.

33. At the feeding time, dead larvae are systematically removed for sanitary reasons. The number of emerged bees and non-emerged bees (pupal mortality) are counted on D22. Adult emergence rate is calculated in percentage by comparing the number of bees emerged on D22 to the number of larvae on D3 when dosing starts. The pupal mortality can be calculated in percentage by comparing the number pupae failed to emerge, including those bees without emergence on D22 and dead pupae removed during pupa stage from D7 to D22) to the number of bees entering pupa stage on D7. The larvae mortality can be calculated in percentage by comparing the number of bees died during larvae stage (from D3 to D7) to the number of larvae on D3 when dosing starts.

34. Other observations, e.g. behavioural, morphological differences including weight (in comparison with controls) should be recorded to aid in the interpretation of mortality data. The presence of uneaten food on D8 should be qualitatively reported.

DATA AND REPORTING

Data and statistical analysis

35. Data are summarised in tabular form, showing for each treatment group, as well as control and reference chemical groups, the number of larvae used, mortalities: larval mortalities from D4 to D8, pupal mortalities from D8 to D15 and D22 (i.e. bees that did not leave their cell). Mortality data are analysed using appropriate statistical methods according to the OECD Guidance Document No. 54 (12).

NOEC assessment

36. The NOEC/D is determined on D22 for adult emergences; it is the highest concentration which, when compared to h the control, has no statistically significant effect ($p<0.05$), within the stated exposure period.

EC_{50} assessment

37. If data allows an EC_{50}/ED_{50}, including its associated lower and upper confidence limits, is calculated on D22 for emerged adult bees. EC_{50}/ED_{50} is the concentration of the test chemical in the diet which, when compared to the control, results in a 50% effect within the stated exposure period.

Test report

38. The test report should include the following:

Test chemical:

- physical nature and relevant physical-chemical properties;
- chemical identification data, including purity of the active ingredient and, if testing a formulated product, the composition of the formulation;

Test species:

- source, species and sub-species of honeybee, supplier of source (if known) and the culture conditions used;
- health condition of the hive used in the test.

Test conditions:

- place and date of the test;
- description of the test system: type of well-plates used, information on the components of the diet (e.g. source of the royal jelly, yeast extract, etc.), number of larvae per treatment level and controls, solvent and concentrations used (if any), test concentrations used for the test chemical;
- incubation conditions: temperature (mean, standard deviation, minimum and maximum values) and relative humidity.

Results:

- the number and percentage of bees considered dead at each treatment level, control(s) and toxic reference chemical (dimethoate or fenoxycarb);
- nominal test concentrations used and measured concentration in the stock solution. The measured concentration should be within 20% of nominal.
- the mortality from D4 to D8, on D15 and D22, NOEC/D and/or EC_{50}/ED_{50} at D22 for adult emergences, and a graph of the fitted model, the slope of the concentration-response curve and its standard error; statistical/mathematical procedures used for the determination of the NOEC/D and EC_{50} if appropriate;
- other observations, including the presence of uneaten food at the end of the feeding period (D7);
- explanation for any deviation from the test method and whether these deviations affected the results.

LITERATURE

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(3) Aupinel, P., Fortini, D., Michaud, B., Marolleau, F., Tasei, J. N., & Odoux, J. F. (2007, Oct 12-14). Toxicity of dimethoate and fenoxycarb to honey bee brood (Apis mellifera), using a new in vitro standardized feeding method. Paper presented at the 9th International Symposium of the ICP-BR-Bee-Protection-Group, York, ENGLAND.

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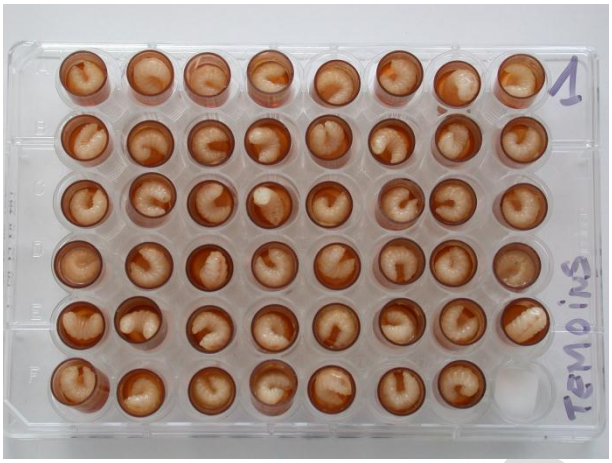
ANNEX

PHOTOS OF PRE-PUPAE (D8) AND PUPAE (D15)



D8

D15